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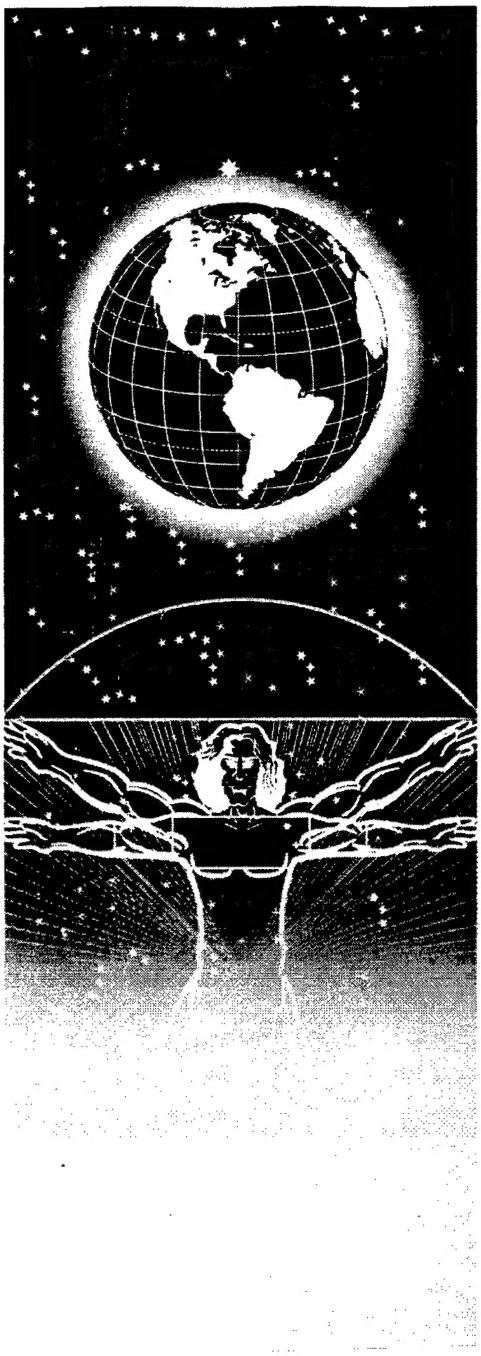

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

Dichloroacetic Acid Metabolism In *Vitro*: I. Investigation of the Factors Influencing Dichloroacetic Acid Metabolism

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TECHNICAL REVIEW AND APPROVAL

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The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

Terry A. Childress

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Dichloroacetic acid (DCA) is a major metabolite of trichloroethylene (TRI), a common groundwater contaminant. Dichloroacetic acid has been reported to cause hepatocarcinomas in rodents. We have shown that DCA metabolism is dependent on a cytosolic protein and, therefore, not P-450 dependent. However, the products of DCA metabolism have not been clearly identified. Initial experiments performed with nuclease and protease ruled out binding of DCA as an explanation for the disappearance of DCA from cytosolic incubations. Experiments were then conducted to determine if a specific cofactor dependence for DCA metabolism existed. Mouse liver cytosol was incubated with either nicotinamide or flavin cofactors at a concentration of 0.9 mM or 0.24-5 mM glutathione (GSH) and with 20-50 µg/ml DCA for 3 to 20 minutes at 37°C. The incubations were derivatized and analyzed to assess DCA removed from solution. Dichloroacetic acid metabolism increased with increasing concentration of GSH. Mouse liver cytosol was then incubated with 0.1-5 mM diethyl maleate (DEM), TCA or monochloroacetic acid (MCA) and 20-50 µg/ml DCA for 5-30 minutes at 37°C. The same analysis was done to calculate µg DCA removed. While TCA appeared to have no effect of DCA metabolism, MCA and DEM had varying effects on DCA metabolism. Results from this research can be used to support further investigation of the products of DCA metabolism.

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PREFACE

Trichloroethylene (TRI), a common industrial solvent, is metabolized to trichloroacetic acid (TCA), trichloroethanol (TCOH), dichloroacetic acid (DCA), and other compounds. Some rodents have developed hepatic tumors after exposure to TRI, TCA and DCA. The focus of this technical report is to describe the metabolism of DCA. The information contained in this report was presented as a poster at the 34th Annual Meeting of the Society of Toxicology in Baltimore, MD, March 1995. This work was supported by DoD Contract No. F33615-90-C-0532 and funded by Strategic Environmental Research and Development Program (SERDP).

The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, DHHS, National Institute of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

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ABBREVIATIONS

C	Celsius
CDNB	Chlorodinitrobenzene
DCA	Dichloroacetic acid
DEM	Diethyl maleate
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GSH	Glutathione
MCA	Monochloroacetic acid
min	Minutes
mM	Millimolar
µg	Microgram
µL	Microliter
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
S9	Cytosol + microsomes + mitochondria
TCA	Trichloroacetic acid
TCOH	Trichloroethanol
TRI	Trichloroethylene

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INTRODUCTION

Dichloroacetic acid (DCA) arises metabolically from trichloroethylene (TRI), a common industrial solvent and a groundwater contaminant (IRP Guide, Vol. 1). DCA is also a byproduct of chlorination. Although TRI itself has not been shown to be directly mutagenic (Greim et al., 1975; Waskell, 1978) it appears that TRI's toxicity is linked to its metabolites.

While DCA has been shown to be carcinogenic, neither its mode of action nor its metabolism have been well studied. Mice have developed hepatocarcinomas after exposure to DCA (Herren-Freund et al., 1987), and rats have shown similar histopathological changes, but not to the same extent (DeAngelo, 1991). DCA has also caused developmental toxicity in rats (Smith et al, 1992). Little is known about the metabolism of DCA beyond the identification of its metabolites (glyoxalate, glycolate, oxalate, CO_2). Rodents given DCA in water by gavage had low, but detectable, blood levels of monochloroacetic acid. Glyoxalate, glycolate and oxalate were identified as urinary metabolites (Larson and Bull, 1992). It has been speculated that DCA is reductively dechlorinated via cytochrome P-450 (Larson and Bull, 1992). However, we have shown that mammalian (rat, mouse, human) DCA degradative activity is localized to the cytosolic subcellular compartment and is therefore, not P-450 dependent (Mahle et al, 1994; Lipscomb et al, 1995). To understand the metabolism of DCA better, experiments were conducted to determine the effects of varying cofactors and potential inhibitors. The results from these experiments are now being used to support the further investigation of DCA metabolism.

METHODS

Hepatic Preparations: B6C3F1 mice were euthanized and their livers perfused *in situ* with ice cold 5 mM Tris/ 154 mM KCl buffer, pH 7.4. Perfused livers were homogenized using a cold glass and teflon homogenizer in 4 volumes of Tris-KCl buffer. Homogenate was centrifuged at 9000 X g for 20 minutes. The resulting S9 fraction was then centrifuged at 105,000 X g for 1 hour. The cytosolic supernatant was re-centrifuged to remove any contaminants. Some cytosolic samples were gel-filtered (de-salted) on a Sephadex G-25 column with a 10,000 MW cutoff (Pharmacia) to remove endogenous salts. Cytosolic protein content was determined using a BCA protein kit from Sigma. The volume of all incubations was 1 mL.

GC Conditions: Incubation media (containing any of the following: cytosol, cofactor, DCA or buffer) was derivatized with dimethyl sulfate, following a modified method of Maiorino et al. (1980). One microliter of derivatized sample was injected on a Hewlett-Packard 5890 GC/ECD fitted with a DB-Wax column (J&W Scientific).

Macromolecular experiments: *Isoquick* nucleic acid extraction kit (Microprobe) was used to determine the amount of DNA and RNA in the cytosol. Cytosol was diluted with buffer to yield 4 mg/ml protein content and pre-incubated with 100 µl of 2 mg/ml protease (Sigma, Type I: Crude from Bovine Pancreas), 100 µl of 0.001 mg/ml DNase

(Boehringer-Mannheim, DNase 1, Grade II) or 100 µl of 0.1 mg/ml RNase (B-M, dry powder) for 30 min at 37°C. After 30 min an NADPH regenerating system and 100 µL of a 200 µg/ml DCA stock solution were added to the samples and incubation continued for 10 min at 37°C. Samples were heat inactivated and analyzed for DCA content by GC.

Cofactor determination:

Flavin - Cytosol and de-salted cytosol at 4 mg/ml protein content were incubated with 30 µg/ml DCA for 3, 5, 10, 15, 20, 25, 30, 35, 40 and 45 min at 37°C. Heat inactivation was used to stop any reaction. A second set of cytosol and de-salted (gel-filtered) cytosol samples was incubated with 30 µg/ml DCA for 25 min. At 25 min 100 µl of 8.8 mM FAD or FMN were added to the samples, and incubation continued for 30, 35, 40 and 45 min. longer. Samples were heat inactivated and analyzed for DCA content by GC.

Nicotinamide - Cytosol (4 mg/ml protein) was pre-incubated with 50 µg/ml DCA for 25 min at 37°C. At 25 min 100 µL of 0.9 mM NAD, NADP, NADH or NADPH was added to each sample and incubation continued for 20 min at 37°C. A control set of cytosol was run without any added cofactor. The samples were heat inactivated and analyzed for DCA content. Cytosol and de-salted cytosol at 4 mg/ml protein content were incubated with 50 µg/ml DCA for 3, 5, 10, 15, 20, 25, 30, 35, 40 and 45 min at 37°C. Heat inactivation was used to stop any reaction. Next, cytosol and de-salted cytosol samples were incubated with 50 µg/ml DCA for 25 min. At 25 min 100 µl of 8.8 mM NADPH were added to the samples, and incubation continued for 30, 35, 40 and 45 min longer. Samples were heat inactivated and analyzed for DCA content by GC.

Glutathione experiments:

A. Two sets of cytosol (4 mg/ml protein) were incubated with 50 µg/ml DCA and 0.25, 1 or 5 mM GSH. One set contained 200 µl of NADPH regenerating system, the other set had no cofactor. The samples were incubated for 3, 6, 10, 20 and 30 minutes at 37°C. Samples were heat inactivated, and DCA was quantitated by GC.

B. Cytosol (4 mg/ml protein) was pre-incubated with 50 µg/ml DCA for 25 min at 37°C. At 25 min an aliquot of DCA was added to yield a concentration of 20 µg/ml, assuming that the majority of the 50 µg/ml was degraded. At the same time 0.5, 1, 2.5 or 5 mM GSH was added to each sample. Incubation continued for 15 min at 37°C. Samples were heat inactivated, and DCA was quantitated by GC.

C. Cytosol (4 mg/ml protein) was pre-incubated with 50 µg/ml DCA for 25 min at 37°C. At 25 min an aliquot of DCA was added to increase remaining DCA concentration to a final concentration of 20 µg/ml DCA. At the same time 0.5, 1, 2.5 or 5 mM GSH, in combination with 5 mM diethyl maleate (DEM) or 0.5 mM chlorodinitrobenzene (CDNB), was added to each sample. Incubation continued for 15 min at 37°C. Control sets were run with 0.5 mM CDNB or 5 mM DEM (both in the absence of GSH) or no addition beyond substrate. Samples were heat inactivated, and DCA was quantitated by GC.

D. Cytosol (4 mg/ml protein) was incubated with 20 μg/ml DCA and either 50 μg/ml TCA or 20 μg/ml MCA. NADPH (0.9 mM) was present as cofactor. Samples were incubated for 10 min at 37°C and heat inactivated. DCA content was quantitated by GC. Cytosol was also incubated with 20 μg/ml DCA and 0.1, 0.5, 1 or 5 mM DEM for 20 min at 37°C. NADPH regenerating system was present as cofactor. Samples were heat inactivated, and DCA was quantitated by GC.

RESULTS and DISCUSSION

The potential for human exposure to DCA is high because of the multiple sources of the carboxylic acid. The mechanism responsible for DCA-induced toxicity is not fully understood; therefore, the focus of these studies was to identify the factors that influenced the metabolism of DCA. Degradation of DCA was measured as disappearance of parent.

After incubation with nuclease or protease, DCA removal from the samples was quantitated, and the results were compared to control samples (Table 1). In both control and nuclease-treated samples approximately 50% of the DCA was degraded, indicating that DNA or RNA do not play a role in the loss of DCA from the cytosolic sample. When cytosol was pre-incubated with protease, less than 5% of the DCA was degraded. This demonstrates that DCA degradation is dependent on cytosolic protein.

Table 1. Cytosolic degradation of DCA. (Results are presented as μg DCA degraded by cytosolic protein, mean ± S.D., n=3)

Treatment	DCA Degraded
Control cytosol	11.1 ± 0.9
DNase-treated cytosol	13.2 ± 0.6
RNase-treated cytosol	12.7 ± 0.07
Protease-treated cytosol	0.48 ± 0.7

After determining that nuclease interaction was not accountable for DCA loss some experiments were performed to determine what factors influenced DCA degradation. Metabolism of DCA in cytosol that was incubated with 30 μg/ml DCA peaked at approximately 20 min, with about 50% of the dose being removed (Fig. 1). An aliquot of the same batch of cytosol was gel-filtered to remove endogenous compounds of low molecular weight. This de-salted cytosol degraded less than 10% of the DCA dose over 30 min of incubation. As the de-salted protein content was identical to non de-salted cytosolic protein content, these results indicate that the removal of some endogenous low-molecular weight component inhibited DCA degradation. The possible argument that the gel-filtering of cytosol damaged its metabolic capability is addressed below. Addition of FAD or FMN to either cytosol or de-salted cytosol did not stimulate DCA degradation.

To determine which (if any) nicotinamide cofactor best stimulated DCA degradation, cytosol was spiked with each of the four nicotinamide cofactors after a 25 min pre-incubation with 50 ug/ml DCA (Table 2, Fig. 2). Incubations containing NADP

and NADPH displayed the greatest DCA loss, both stimulating the degradation of approximately 75% of the dose. Degradation of DCA by de-salted cytosol was evaluated under the same conditions with NADPH as cofactor. (Data not shown). De-salted cytosol degraded approximately 25% of the dose; the addition of NADPH at 25 min increased degradation by about 20%. The mechanism for this stimulation is as yet undetermined. For the remaining experiments NADPH or NADPH regenerating system was included.

Table 2. Cytosolic degradation of DCA. (Results are presented as μg DCA degraded by cytosolic protein, mean ± S.D, n=3.)

Treatment	DCA degraded
NAD	11.1 ± 0.5
NADP	37.3 ± 0.8
NADH	14.7 ± 1.7
NADPH	34.5 ± 1.9

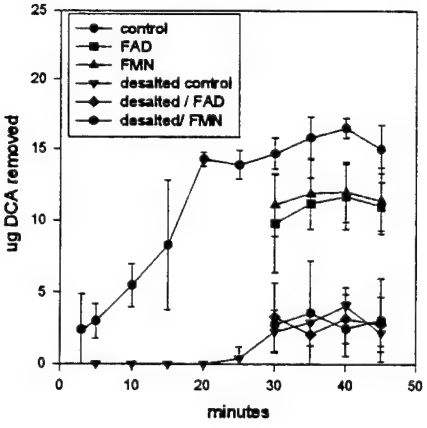

Figure 1. Effect of flavin cofactors on DCA degradation.

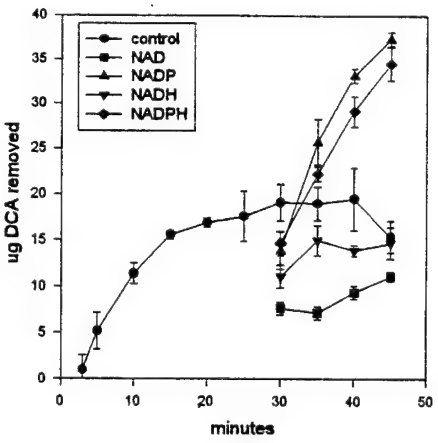

Figure 2. Effect of nicotinamide cofactors on DCA degradation.

Since NADPH increased DCA degradation and NADPH is involved in the reduction of oxidized glutathione (a major anti-oxidant), we sought to determine whether GSH influenced DCA metabolism. After cytosol was incubated with 50 ug/ml DCA and GSH (with and without nicotinamide cofactor), the amount of DCA removed from the samples was quantitated (Fig. 3 a, b, c). Cytosol incubated with 0.25 mM GSH (with and

without cofactor) degraded approximately 6 % of the DCA after 6 min as compared to the control. Samples incubated with 1 and 5 mM GSH showed essentially no difference between the group containing cofactor and the group without cofactor. Cytosol incubated with 1 and 5 mM GSH degraded an average of 8% and 9.5% more DCA than control, respectively. Essentially, the addition of GSH did not increase the metabolism of DCA with or without NADPH.

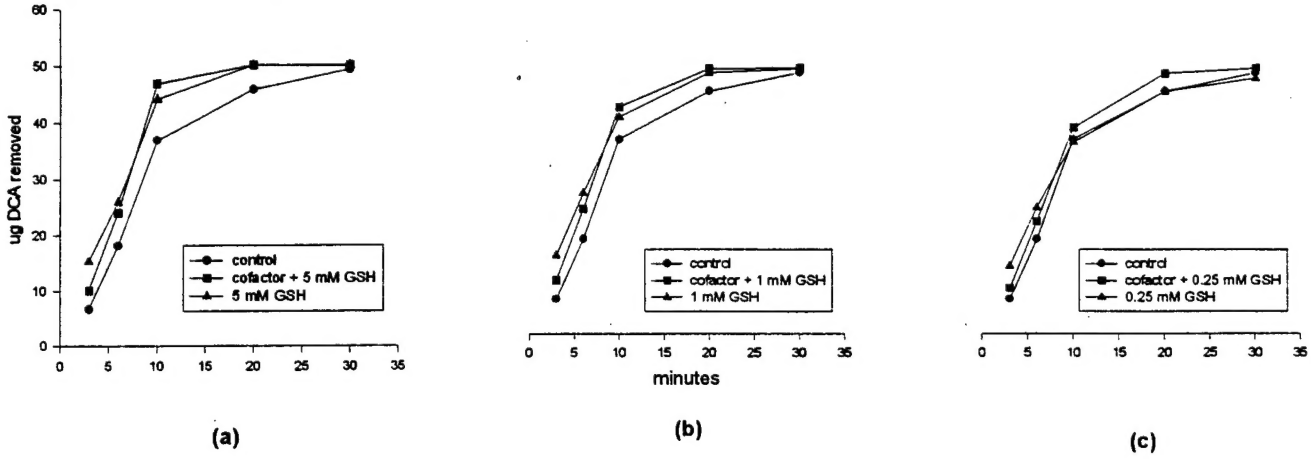

Figure 3. Effect of glutathione on DCA degradation a) 5 mM, b) 1 mM, c) 0.25 mM.

To further investigate the contribution of GSH to DCA degradation, cytosolic samples were pre-incubated with 50 μg/ml DCA for 25 min and then spiked to yield 20 μg/ml DCA and increasing concentrations of GSH (Fig. 4). Removal of DCA increased with increasing GSH concentration. To challenge the effect of GSH, cytosolic samples were incubated with CDNB which enzymatically conjugates with GSH or DEM which depletes GSH by non-enzymatic conjugation. Cytosolic samples that were incubated with only DEM or CDNB degraded approximately the same amount DCA. Methanol was run as a vehicular control for CDNB. Samples that were incubated with GSH and DEM or CDNB removed approximately 41 μg of DCA.

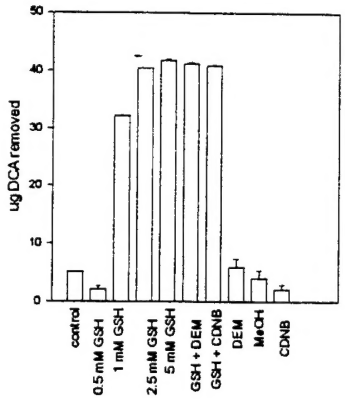

Figure 4. Glutathione effect on DCA degradation.

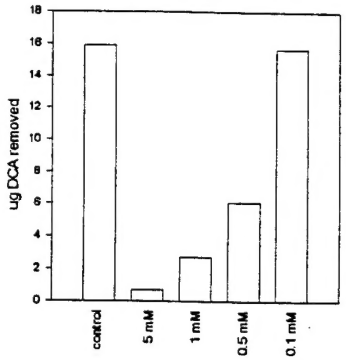

Figure 5. Diethyl maleate effect on DCA degradation.

The effect of DEM-induced GSH depletion on DCA degradation was further investigated. After incubation of cytosol with 20 μg/ml DCA and increasing concentrations of DEM, the removal of DCA was quantitated (Fig. 5). DCA degradation in the presence of DEM demonstrated a clear dose-dependent decrease, which was evident at 0.1 mM DEM. Although GSH increases the removal of DCA, even in the presence of GSH depletors, the mechanism is unclear.

Since MCA, TCA and DCA are products of TRI metabolism, we attempted to determine if the presence of TCA or MCA influenced metabolism of DCA. Cytosolic degradation of DCA in the presence of 50 μg/ml TCA (Table 3) equaled that of the control; TCA did not inhibit DCA metabolism. Cytosolic samples containing 20 μg/ml MCA degraded only 1.5 μg DCA; the presence of MCA inhibited the breakdown of DCA. As extremely low levels of MCA are observed *in vivo,* it is unclear whether this *in vitro* effect is predictive of the *in vivo* situation. Future experiments should elucidate the potential impact of physiologically relevant concentrations of MCA on DCA degradation.

Table 3. Inhibition of cytosolic DCA degradation. (Results are presented as μg DCA degraded by cytosolic protein, mean ± S.D., n=3.)

Treatment	DCA degraded
DCA control	11.7 ± 0.2
DCA + TCA	11.5 ± 0.4
DCA + MCA	1.5 ± 1.1

There is on-going work to understand the metabolism of TRI and DCA and the link between metabolism and toxicity. The goal of these studies was to investigate the effect of enzyme systems, cofactors and inhibitors on DCA metabolism. The findings of these studies help clarify the degradation of DCA in cytosolic systems and are being used to support further investigation of the products of DCA metabolism.

CONCLUSIONS

- DNA and RNA interaction does not play a role in DCA loss.
- Of the nicotinamide cofactors, NADP and NADPH, best stimulate DCA degradation
- GSH stimulates DCA degradation through an unknown mechanism.
- Compounds which deplete GSH decrease the amount of DCA degraded by cytosol. This effect is negated in the presence of exogenous GSH.

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